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Glatiramer acetate attenuates the pro-migratory profile of adhesion molecules on various immune cell subsets in multiple sclerosis

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Summary

An altered expression pattern of adhesion molecules (AM) on the surface of immune cells is a premise for their extravasation into the central nervous system (CNS) and the formation of acute brain lesions in multiple sclerosis (MS). We evaluated the impact of glatiramer acetate (GA) on cell-bound and soluble AM in the peripheral blood of patients with relapsing–remitting MS (RRMS). Fifteen patients treated *de novo* with GA were studied on four occasions over a period of 12 months. Surface levels of intracellular cell adhesion molecule (ICAM)-1, ICAM-3, lymphocyte function-associated antigen (LFA)-1 and very late activation antigen (VLA)-4 were assessed in T cells ($CD3^+CD8^+$, $CD3^+CD4^+$), B cells, natural killer (NK) cells, natural killer T cells (NK T) and monocytes by five-colour flow cytometry. Soluble E-selectin, ICAM-1, ICAM-3, platelet endothelial cell adhesion molecule (PECAM)-1, P-selectin and vascular cell adhesion molecule (VCAM)-1 were determined with a fluorescent bead-based immunoassay. The pro-migratory pattern in RRMS was verified by comparison with healthy controls and was characterized by up-regulation of LFA-1 ($CD3^+CD4^+$ T cells, B cells), VLA-4 ($CD3^+CD8^+$ T cells, NK cells), ICAM-1 (B cells) and ICAM-3 (NK cells). Effects of GA treatment were most pronounced after 6 months and included attenuated levels of LFA-1 ($CD3^+CD4^+$) and VLA-4 ($CD3^+CD4^+$, $CD3^+CD8^+$, NK, NK T, monocytes). Further effects included lowering of ICAM-1 and ICAM-3 levels in almost all immune cell subsets. Soluble AM levels in RRMS did not differ from healthy controls and remained unaltered after GA treatment. The deregulated pro-migratory expression profile of cell-bound AM is altered by GA treatment. While this alteration may contribute to the beneficial action of the drug, the protracted development and unselective changes indicate more secondary immune regulatory phenomena related to these effects.

Keywords: adhesion molecule, glatiramer-acetate, immunomodulation, multiple sclerosis, transmigration

Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder of the central nervous system (CNS) which is believed to result from environmental exposure in genetically susceptible individuals [1]. Recent studies have attributed impaired suppressive activity of regulatory T cells (T_{regs}) and subsequent expansion of myelin-reactive T helper type 1 (Th1) and Th17 cells in the pathogenesis of the disease [2]. Trafficking of immune cells from the systemic compartment across the blood–cerebrospinal fluid (CSF) barrier or the blood–brain barrier (BBB) is a critical step in the pathogenesis of the disease. The neuropathological correlates in relapsing–remitting MS (RRMS) are recurrent episodes of focal myelin degradation, followed by complete or partial remyelination [3].

Glatiramer acetate (GA, Copaxone®) is a synthetic, random co-polymer used widely as a first-line agent for

the treatment of RRMS. The agent was developed initially to mimic a major component of the myelin sheet, but unexpectedly inhibited development of disease in experimental allergic encephalomyelitis (EAE), an animal model of MS. Randomized controlled clinical trials confirmed the clinical efficacy of GA in RRMS comparable to the other first-line substance, interferon (IFN)-β [4]. The corresponding effects on neuroimaging include a lower size and frequency of Gadolinium-enhancing lesions [5,6]. The action of GA had been attributed initially to competition for binding to major histocompatibility complex type II molecules on the surface of antigen-presenting cells with other putative antigens, and subsequent reduced activation of encephalitogenic T cells [7]. Further immunomodulatory actions include preferential differentiation of $CD4^+$ T cells into anti-inflammatory Th2 phenotype, increased frequency and function of $CD25^+$forkhead box protein 3 $(FoxP3)^+$ T_{regs}, modulation of $CD8^+$ T cells and expansion of regulatory type II monocytes [7]. Several reports have demonstrated that lymphocytes from patients with RRMS have increased migratory properties. This pro-migratory capacity was shown to depend upon the up-regulation of chemokine receptors, secretion of matrix-metalloproteinases and binding of leucocyte integrins to their endothelial ligands. The adhesion molecules (AM) implicated in MS are very late activation antigen-4 (VLA-4) (α4β1, CD49d/CD29) of the β1 integrin family and the β2 integrin lymphocyte function-associated antigen-1(LFA-1) (αLβ2, CD11a/CD18) on immune cells and their ligands, vascular cell adhesion molecule-1 (VCAM-1, CD106) and intercellular cell adhesion molecule-1 (ICAM-1, CD54) [8]. Histopathological studies have revealed that endothelial ICAM-1 and VCAM-1 and their respective ligands LFA-1 and VLA-4 on infiltrating immune cells are up-regulated in acute MS lesions [9]. Patients with MS have at least a 1·5 to threefold higher expression of VLA-4 and LFA-1 on the surface of peripheral blood mononuclear cells (PBMC) [10]. Some of the mechanisms of action of GA apparently interfere with leucocyte transmigration. Prat and co-workers, for instance, reported that immune cells derived from IFN-β or GA-treated patients with RRMS had lower migratory capacity compared to untreated patients [11]. Moreover, the impact of supernatants from GA-reactive Th2-polarized T cells on expression levels of endothelial AM was less pronounced compared to GA-reactive Th0/Th1 polarized T cells [12]. Previous studies focused mainly on the characterization of AM on the surface of T cells, B cells and monocytes. Natural killer (NK) cells and natural killer T cells (NK T) are lymphocyte subsets but, like monocytes, are players of the innate immune system and implicated in cancer, infection and autoimmunity [13,14]. Soluble forms of AM gained attention not only as markers of endothelial activation; elevated serum levels of sICAM-1 and sVCAM-1 were shown to be associated with BBB disruption and correlated with clinical and magnetic resonance imaging (MRI) activity [15,16]. Notably, soluble P-selectin, soluble platelet endothelial cell adhesion molecule (sPECAM)-1 and soluble E-selectin have been proposed as surrogate markers of disease activity in RRMS [17].

The purpose of this study was to investigate the impact of GA treatment on cell-bound AM of selected PBMC subsets representative of both innate and adaptive immunity from RRMS patients. In a second series of experiments we assessed whether or not treatment with GA alters soluble AM levels in serum of patients with RRMS.

Material and methods

Patients

The study was approved by the local ethics committee (Ethikkommission Bundesland Salzburg 415-E/984/2-2008) and all patients gave written consent. Patients with clinically definite RRMS based on the McDonald criteria of 2005 [18] and assigned *de-novo* to GA treatment were recruited [$n = 15$, 12 female, mean age 41·1 years, standard deviation (s.d.) 6·2]. The minimum interval from the last relapse to the initiation of GA treatment was 4 weeks. Adults with no signs or symptoms of an immunological or inflammatory illness constituted the group of healthy controls (HC, $n = 19$, 13 female, mean age 44·8 years, s.d. 12·7).

Sample collection and preparation

Peripheral venous blood was collected in PBMC enrichment (Becton Dickinson AG, Basel, Switzerland) or serum gel blood collection tubes (Sarstedt AG, Nümbrecht, Germany) at predefined time-points [before initiation of GA treatment and after 1·5 ($n = 14$), 6 ($n = 13$), 9 ($n = 12$) and 12 months ($n = 9$)]. PBMC enrichment was performed as described recently [19]. For the study of soluble AM, serum was collected after centrifugation at 2000 ***g*** for 10 min. Aliquots were stored at −20°C until further processing.

Determination of cell-bound AM on immune cell subsets

Surface levels of ICAM-1 [RR1/1, fluorescein isothiocyanate (FITC); eBioscience, Vienna, Austria], ICAM-3 (CBR-IC3/1, FITC; eBioscience), CD11a/LFA-1 (25·3, FITC; alpha-L subunit of LFA-1) and CD49d/VLA-4 (clone HP2/1, FITC; alpha-4 subunit of VLA-4) on seven immune cell subsets [$CD3^+$ T cells (clone UCTH1, ECD), $CD3^+CD4^+$ T cells (SFCI12t4D11, PC7), $CD3^+CD8^+$ T cells (B9·11, PC5), $CD19^+$ B cells (J4·119, PC7), $CD14^+$ monocytes (RMO52, PC5), $CD56^+CD16^+$ NK cells (clones NKH-1 and 3G8, both phycoerythrin (PE)] and

$CD56^+CD16^+CD3^+$ NK T cells expressed as relative fluorescence intensities (RFI) were analysed by five-colour flow cytometry (Cytometrics FC500; Beckman Coulter, Vienna, Austria), as described recently [19]. For improved inter- and intra-individual comparability, RFI levels were calculated from median fluorescence intensities (MFI) of the single immune cell subpopulations by correcting them for the MFI of negative isotype-matched antibodies [immunoglobulin (Ig)G1-FITC/PE (clone ZX-3, Exalpha, Watertown, MA, USA), IgG-ECD/PC5/PC7 (clone 679·1Mc7)] and relating them to the RFI of the positive controls [CD45-FITC/PE/ECD/PC5/PC7 (clone J33)] [20]. The calculation was as follows: MFI (test sample) − MFI (isotype control)/MFI (positive control) − MFI (isotype control) × 1000 (annotation: multiplication by 1000 in reference to the log scale). Unless specified otherwise, all antibodies were obtained from Beckman Coulter.

Determination of soluble AM

The human adhesion 6plex FlowCytomix Multiplex kit (eBioscience) was used for measuring serum concentrations of soluble AM [E-selectin (endothelial leucocyte adhesion molecule-1, ELAM-1), sICAM-1, sICAM-3, sPECAM-1 (CD31), P-selectin (CD62P, GMP-140) and sVCAM-1 (CD106)], according to the manufacturer's instructions. The prespecified detection limits were as follows (in ng/ml): sICAM-1 (5·3), SE-selectin (1·2), sICAM-3 (4·8), sPECAM-1 (0·8), sVCAM-1 (0·2) and sP-selectin (5·7).

Statistics

The GraphPad Prism version 5·0 program (GraphPad Prism Software Inc., San Diego, CA, USA) was used for statistics and preparation of graphs. As none of the data sets were distributed normally, non-parametric tests were used for all analyses. A P-value $\leq 0{\cdot}05$ was considered to represent a statistically significant difference.

Results

Surface expression of AM in RRMS and HC

The four cell-bound AM (ICAM-1, ICAM-3, LFA-1, and VLA-4) were detected on all seven investigated immune cell subsets, although at different levels. To substantiate the proposed disease-related deregulation of AM expression patterns in MS, we compared AM levels of immune cells from HC ($n = 19$) with those from treatment-naive MS patients ($n = 15$, Fig. 1). In MS patients, ICAM-1 levels were increased significantly on the surface of B cells ($P < 0{\cdot}05$), ICAM-3 on NK cells ($P < 0{\cdot}01$), LFA-1 ($P < 0{\cdot}01$) on $CD4^+$ T cells [which was also reflected in the $CD3^+$ T cell population, both ($P < 0{\cdot}01$)] and on B cells ($P < 0{\cdot}05$). VLA-4 levels increased significantly on the surface of $CD8^+$ T cells ($P < 0{\cdot}05$) and $CD19^+$ B cells ($P < 0{\cdot}05$). No differences in AM expression levels were observed on NK T cells or monocytes from MS patients and HC.

Effects of GA on AM expression levels in RRMS

To determine the potential impact of GA therapy on cell-bound AM, the surface expression of ICAM-1, ICAM-3, LFA-1 and VLA-4 were studied on the immune cell subsets at different time-points (1·5, 6, 9 and 12 months from treatment initiation) during a period of 12 months and related to baseline levels (Fig. 2). A decrease in the expression levels of ICAM-3 was found on all immune cell subsets following treatment with GA. Similar results were observed for ICAM-1 with GA treatment, with B cells, $CD8^+$ T cells and monocytes being the only exceptions. ICAM-1 expression was unaltered on B cells over the entire observation period. In contrast, ICAM-1 even increased on monocytes and $CD8^+$ T cell after 9 and 12 months of treatment, respectively.

The time-point for the observed decreases in AM expression differed among the immune cell subsets but persisted until the end of the observation period (9–12 months). Detailed analysis revealed early (at the 1·5-month examination) and late (after >6 months) effects. ICAM-3 surface levels, for example, were decreased early in the course on $CD4^+$ and $CD8^+$ T cells, NK T cells and monocytes. In contrast, decreases in ICAM-1 on $CD4^+$ T cells were seen after 6 months of treatment, and 12 months on NK and NK T cells. We observed an early decrease in VLA-4 expression levels on $CD4^+$ T cells and a later effect with decreased levels after 6 months on $CD8^+$ T cells, NK cells and monocytes, and after 12 months on NK T cells. VLA-4 expression on the surface of B cells remained unaltered during GA treatment. LFA-1 surface levels were found to be decreased on only $CD4^+$ T cells after 9 months of treatment with GA, and unchanged in other immune cell subsets.

Soluble AM in RRMS and HC

Six soluble AM (sE-selectin, sICAM-1, sICAM-3, sPECAM-1, sP-selectin and sVCAM-1) were investigated; all had detectable levels in the serum of patients with MS ($n = 15$) and healthy controls ($n = 19$). The medians (range) in MS patients and HC were as follows (in ng/ml): sE-selectin [128·8 (54·8, 196·4); 125·8 (34·0, 274·8)], sICAM-1 [490·3 (300·5, 1419·0); 542·2 (388·4, 1833·0)], sICAM-3 [73·7 (12·7, 130·9); 82·2 (43·4, 133·9)], sPECAM-1 [164·0 (115·7, 215·1); 159·1 (120·4, 276·1)], sP-selectin [651·6 (262·7, 1114·0); 668·1 (209·1, 1022·0)] and sVCAM-1 [599·0 (473·1, 1707·0); 708·1 (414·7, 2161·0)]. Statistical analysis did not disclose any significant differences between the serum concentrations of the two cohorts [non-significant (n.s.)].

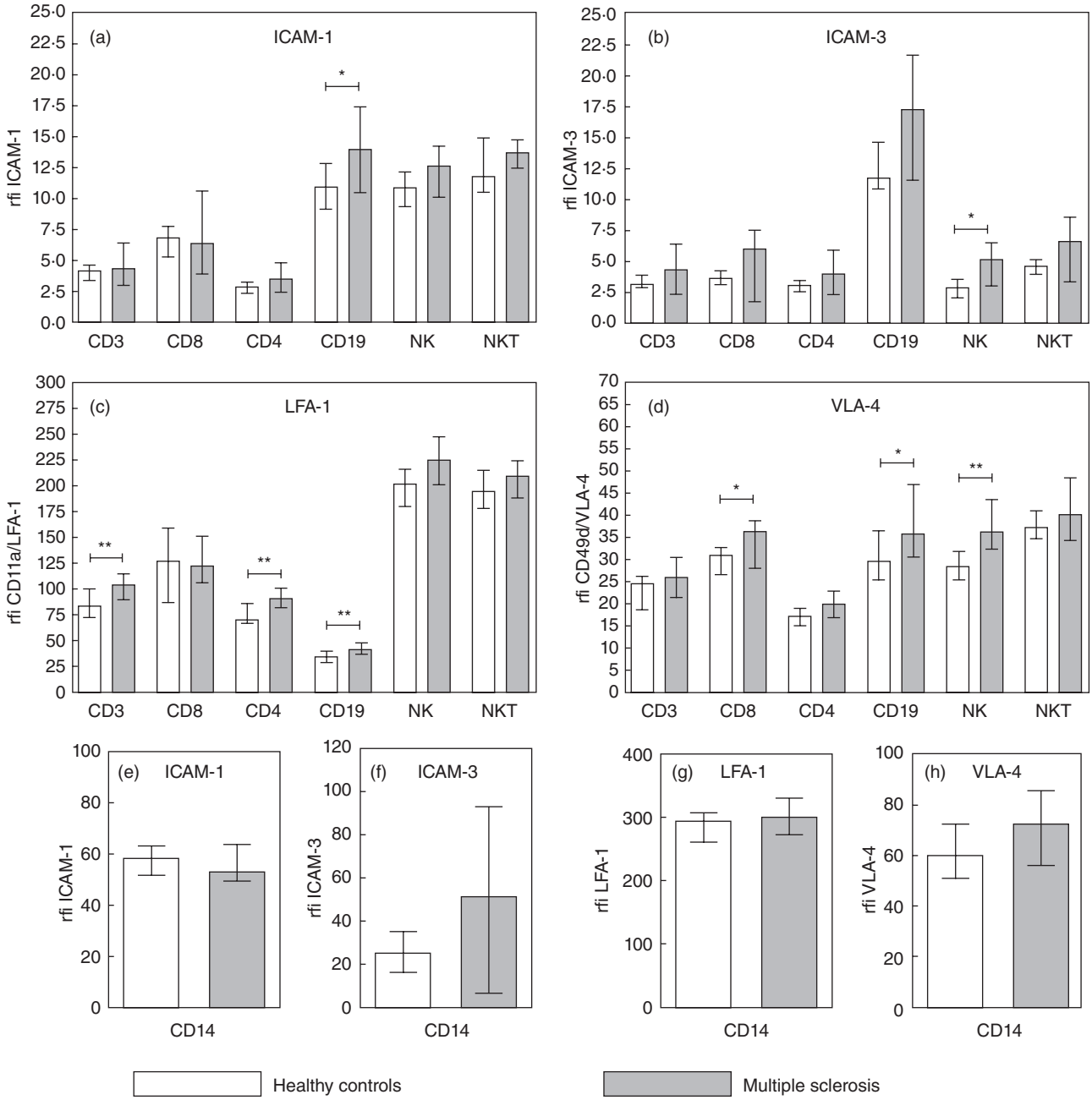

Fig. 1. Cell-bound adhesion molecules (AM) on different mononuclear cell populations from treatment-naive patients with relapsing–remitting multiple sclerosis ($n = 15$) and healthy controls ($n = 19$) (a–h). The box-plots represent medians and the errors bars the interquartile range (25th and 75th percentiles). $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$; mo.: months.

Effects of GA on soluble AM in RRMS

Serum concentrations of the six soluble AM (sE-selectin, sICAM-1, sICAM-3, sPECAM-1, sP-selectin and sVCAM-1) were investigated longitudinally after 1·5, 6, 9 and 12 months of GA treatment and compared to baseline levels. No treatment effect of GA on serum concentrations of the six soluble AM was observed during the 12-month study period (Table 1).

Discussion

The expression profile of AM in MS

The firm adhesion of encephalitogenic T cells and other leucocyte subsets to CNS endothelial cells and ependymal cells is critically dependent upon the binding of AM to their respective receptors. A number of studies have linked the increased migration rate of T cells isolated from MS

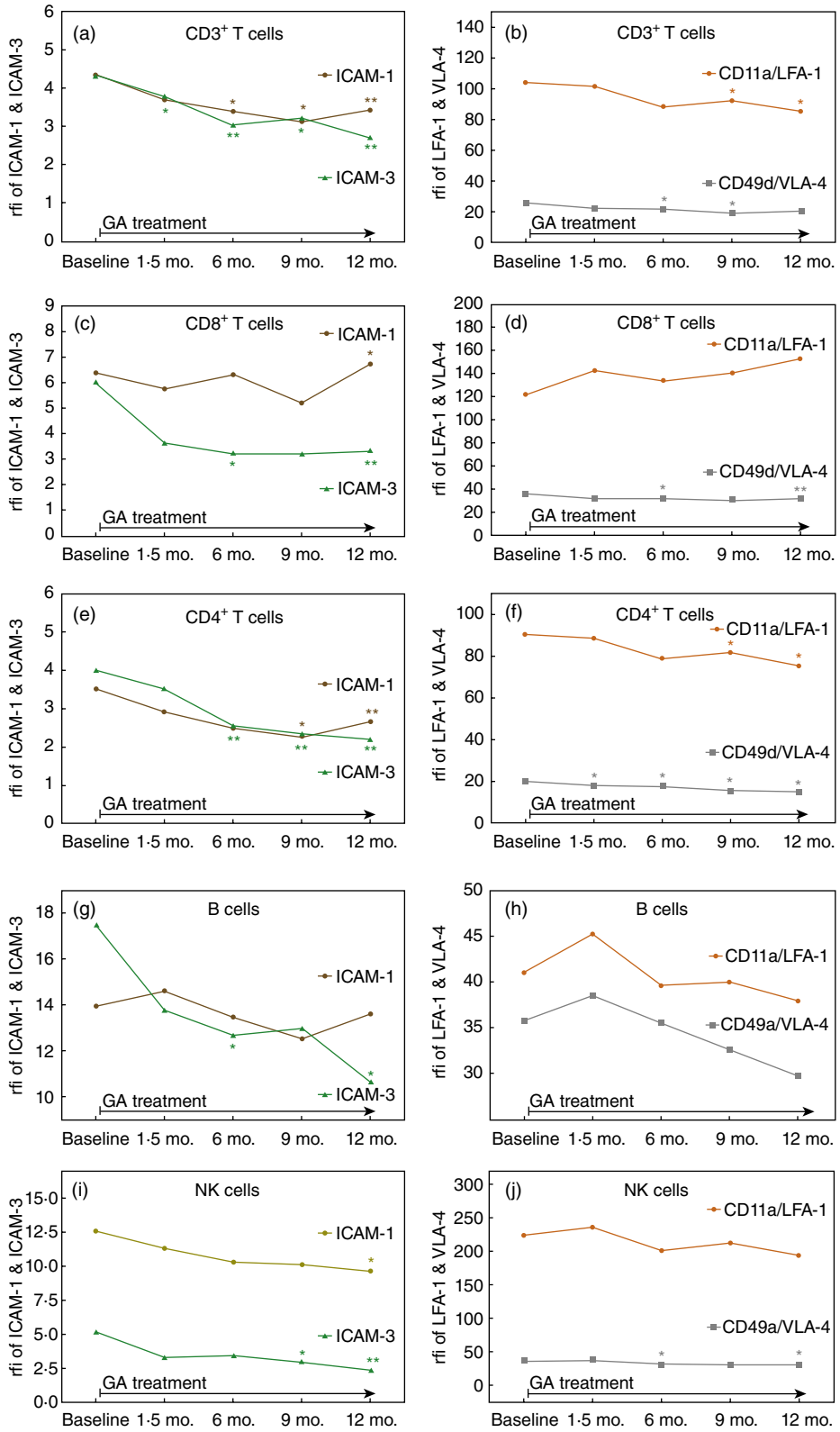

Fig. 2. Effect of glatiramer acetate (GA) treatment on cell-bound adhesion molecules (AM) on the surface of different mononuclear cell populations was assessed at four time-points over a period of 12 months from treatment initiation (a–n). The dots at each time-point indicate the median. There were 14 patients at 1·5 months, 12 at 6 months, 11 at 9 months 10 at 12 months. Intracellular cell adhesion molecule (ICAM)-1 (brown), ICAM-3 (green), lymphocyte function-associated antigen (LFA)-1 (orange) and very late activation antigen (VLA)-4 (grey). $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

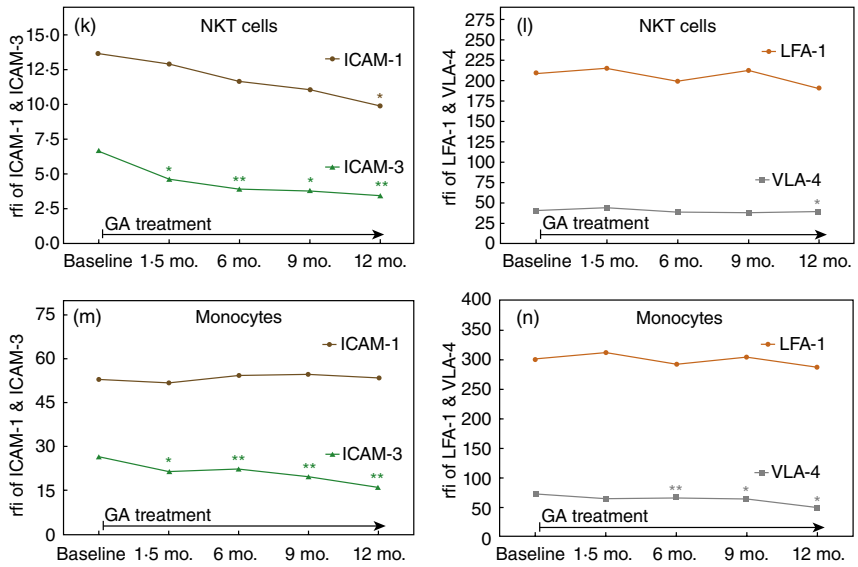

Fig. 2. *Continued*

patients to the higher surface expression of LFA-1 and VLA-4 on lymphocytes [21–23]. Our data corroborate this idea of linking pro-migratory immune cell activity with surface-bound AM expression levels, which are increased in treatment-naive patients with RRMS compared to healthy controls. Increased surface levels of cell-bound AM were identified on $CD4^+$ T cells (LFA-1), $CD8^+$ T cells (VLA-4), NK cells (ICAM-3) and $CD19^+$ B cells (ICAM-1, VLA-4). Various immune cell subsets are present in MS lesions, with $CD4^+$ T cells predominating in acute lesions and $CD8^+$ T cells in chronic lesions [24]. Most recently, it was shown in EAE that Th1 cells infiltrate the spinal cord preferentially via a VLA-4-mediated mechanism, whereas entry of Th17 cells depends upon LFA-1 [25]. The data regarding increased VLA-4 on $CD8^+$ T cells in MS are in line with a study by Jensen and co-workers [22]. While our data show that LFA-1 is increased on $CD4^+$ T cells and VLA-4 on $CD8^+$ T cells in MS, other studies did not detect such a deregulation [26,27]. Most recently, a reduction of NK cells was shown to precede the development of relapses and brain lesions [28]. Hamann and co-workers reported a decreased frequency of this cell type in the CSF of MS patients and speculate that the CSF is an intermediary compartment for NK cell trafficking and differentiation before entering the CNS parenchyma [14]. The increased expression of ICAM-3 on the surface of NK cells in peripheral blood found in this study may support this process. While B cells constitute only a minor subset of immune cells in normal CSF, the intrathecal accumulation of clonally expanded B cells is a prevailing observation in MS [29,30]. Cells of the B cell lineage within the CNS compartment and synthesis of immunoglobulins have been linked to up-regulation of AM in active MS plaques and increased levels of the chemokines CXCL12 and CXCL13 in the CSF [31,32]. B cells were shown to have a higher migratory capacity than T cells [33]. Memory B cells ($CD27^+$), the predominant B cell subset in CSF of MS patients, have a high expression of VLA-4 in contrast to naive B cells ($CD27^-$) [34,35]. An interesting

Table 1. Effect of glatiramer acetate (GA) over a treatment period of 12 months on serum concentrations of six soluble adhesion molecules [AM) (sE-selectin, soluble intracellular cell adhesion molecule (ICAM)-1, sICAM-3, soluble platelet endothelial cell adhesion molecule (PECAM)-1, sP-selectin and soluble vascular cell adhesion molecule (VCAM)-1].

	Baseline ($n = 15$)	1·5 months ($n = 14$)	6 months ($n = 12$)	9 months ($n = 11$)	12 months ($n = 10$)
sE-selectin	128·8 (54·8, 196·4)	86·5 (33·6, 172·4)	94·9 (32·8, 179·2)	86·2 (32·4, 172·4)	91·4 (39·4, 193·5)
sICAM-1	388·4 (542·2, 1833·0)	593·2 (328·0, 1288·0)	591·3 (307·1, 1295·0)	647·2 (279·1, 1155·0)	720·2 (305·5, 1131·0)
sICAM-3	73·7 (12·7, 130·9)	56·9 (18·1, 175·4)	46·1 (26·7, 224·5)	58·5 (23·2, 242·7)	50·8 (27·8, 158·9)
sPECAM-1	164·0 (115·7–215·1)	130·8 (41·3, 252·4)	133·2 (40·7, 264·6)	78·3 (45·7, 189·2)	102·5 (39, 208·5)
sP-selectin	651·6 (262·7, 111·0)	652·7 (311·1, 896·4)	719·9 (156·6, 917·6)	747·4 (211·5, 1320)	752·2 (250·9, 1120)
sVCAM-1	599·0 (473·1, 1707·0)	482·6 (284·0, 725·6)	429·5 (243·1, 654·2)	389·9 (249·7, 612·1)	486·8 (297·7, 840·5)

Median (range) in ng/ml.

finding in our study was the increased expression of ICAM-1 and VLA-4 on the surface of $CD19^+$ B cells in untreated RRMS patients. With regard to increased VLA-4 surface levels, we now confirm our previous observation in a larger cohort [19]. This alteration in AM expression is of functional relevance, as antibodies against ICAM-1 and VLA-4 but not VCAM-1 were able to inhibit the migration of B cells across human bronchial epithelial cells (HBEC) [33].

In contrast to the analysis of cell-bound AM, the levels of the six soluble AM in MS patients were in the same range as in the controls. This is in line with the most recent studies for serum or plasma levels of sVCAM-1 and sICAM-1 in RRMS [36,37]. However, there are studies reporting a correlation of these molecules with disease course and subtype, notably in the examination of CSF [38]. The comparatively low numbers of patients, the analysis of serum and the remission phase on study entry may have impeded the examination. Indeed, studying a larger number of patients (RRMS $n = 98$), Kuenz and co-workers reported that plasma concentrations of three soluble AM (sPECAM-1, sP-selectin and sE-selectin) were highest in RRMS compared to primary ($n = 15$) and secondary progressive MS ($n = 53$), and increased even further during relapse [17].

The impact of GA on cell-bound and soluble AM in MS

Importantly, we demonstrate that treatment with GA results in a decreased surface expression of the AM linked to the pro-migratory activity of PBMCs. GA induced an overall decline of ICAM-1 and ICAM-3 expression in the majority of the immune cell subsets studied. This finding adds to the current knowledge of phenomena concerning the down-regulation of AM following therapy with immunosuppressive agents such as methylprednisolone and immunomodulatory treatment with IFN-β [10,39]. However, the time–course of changes with GA contrasts with observations concerning steroids, where a rapid change is observed. The impact of GA on LFA-1 and VLA-4 levels was more selective than for ICAMs. LFA-1 surface expression decreased only on $CD4^+$ T cells following GA treatment. Significantly lower levels of VLA-4 were detected in the course of GA treatment on NK cells, NK T cells and monocytes. A more profound effect on VLA-4 was reported with natalizumab therapy, and included the impact on all leucocyte subsets referred to in this study [19,40]. This humanized antibody directed against the α4β1 integrin receptor was shown to reduce lesion formation and disease activity effectively. Natalizumab therapy, however, was also associated with lower LFA-1 levels on B cells and ICAM-2 levels on B cells and monocytes, indicating secondary phenomena associated with blocking of VLA-4 [41]. The ICAM-3 expression levels on $CD19^+$ B cells in our study, regardless of MS or HC, was at least twice as high as in other immune cell subsets. We have reported previously the decrease of ICAM-3 on T and B cells following therapy with IFN-β, and now show a profound impact of GA on ICAM-3 expression levels [39]. Several lines of evidence indicate that GA may affect cellular B cell function. GA treatment was shown to limit the expansion of myelin-reactive Th1 and Th17 cells by interfering with B cells as antigen-presenting cells and the promotion of regulatory B cells [7,42]. The generation of GA-specific Th2 cells secreting anti-inflammatory cytokines might limit the activation of B cells. The induction of regulatory B cells with provision of anti-inflammatory cytokines and interference with their role as antigen-presenting cells might participate in the altered AM expression [43,44]. In contrast, the levels of the four soluble AM in MS patients were in the same range compared to controls and remained unchanged following treatment with GA.

Some of the immunological effects induced by GA are observed as early as 1·5 months after initiation of treatment and decline after 6 months [45,46]. With observation of the attenuation of surface levels mainly not before 6 months from treatment initiation, the effect by GA seems to occur indirectly and is likely to be a result of secondary regulatory processes. This is also supported by studies from Kim and co-workers, which disclosed that GA treatment suppresses the inflammatory potential of T cells and subsequently inflammatory responses at the BBB, but not directly their migratory capacity [12]. Further evidence comes from the persistence of the effects on cell-bound AM, as shown in our study. There are reports that most of the immunological effects, particularly the Th2 bias and GA-reactive T cells, do not diminish over time [47]. Of note, there might be interindividual differences related to the pathogenesis, as seen with the effect of GA on the generation of GA-reactive and Th2-polarized cells. Because of the limited number of patients and lack of scheduled MRI scans, we did not take the individual clinical and neuroradiological course into account. By using the fluorescence activated cell sorter (FACS) method we did not account for conformational changes and clustering of AM. Further studies should consider emerging adhesion molecules, including ninjurin-1, activated leucocyte cell adhesion molecule (ALCAM, CD166) and melanoma cell adhesion molecule (MCAM).

Conclusion

To our knowledge, this is the first study in MS to characterize a panel of cell-bound AM on different subsets of mononuclear cells together with their soluble form and following treatment with GA. We found evidence that GA treatment is associated with modulation of the AM profile and thus the pro-migratory AM pattern of different immune cell subsets. While there is evidence that these phenomena are related to secondary immune regulation processes, these effects may contribute partially to the clinical and MRI effects seen in patients with MS.

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